FUNCTIONAL IMPACT OF β, β-CAROTENE-9', 10'-OXYGENASE 2 (BCO2) IN HEPATIC MITOCHONDRIA

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Abstract: β, β-carotene-9', 10'-dioxygenase 2 (BCO2) is a carotenoid cleavage enzyme in the inner membrane of mitochondria. However, BCO2 is inactivated in human macula where xanthophyll is preferentially accumulated. The purpose of this study is to determine whether intact BCO2 protein impacts integrity of hepatic mitochondrial function in mice. Male BCO2 knockout (KO) and the genetic background wild type (WT) mice, at 6 weeks of age, were used. Isolated hepatic mitochondria were subjected to mitochondrial proteome profiling (by LC-MS/MS) and functional integrity assay (by the assay of coupling and electron flow). Liver tissue metabolomics was also explored. The basal respiratory rate, the proton leak and the maximal ATP production were all significantly elevated in the BCO2 knockout liver compared to wild type liver. The capacities of mitochondrial complex II and complex IV was greater in knockout mice than that in the wild type mice. Mitochondrial proteomic profiling results indicated alterations of enzymes and proteins involved in fatty acid β–oxidation, the tricarboxylic acid cycle, ETC/oxidative phosphorylation, and the metabolism of carotenoids. The global metabolic profiles showed that increased level of oxidative stress and alterations of lipid metabolism in BCO2 knockout liver compared to wild type liver. In conclusion, BCO2 is essential to maintain the hepatic mitochondrial normal function.

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CHAPTER I

INTRODUCTION

Carotenoids are 40-carbons lipophilic pigments, which can be synthesized in plants, bacteria and fungi, and play important roles in animal and plant physiology, such as being a vitamin A precursor [1]. Carotenoids can be classified into two groups: the xanthophyll, which contain oxygenated groups and the carotenes, which are only hydrocarbons without oxygenated groups and less polar than the xanthophyll [2, 3]. β, β-carotene-9', 10'-oxygenase 2 (BCO2, also named BCDO2 and CMO2) and β, β-carotene-15, 15'-monooxygenase 1 (BCMO1) are the two cleavage enzymes that are identified to play important roles in carotenoids metabolism [4]. BCMO1 symmetrically cleaves pro-vitamin A carotenoids, such as β-carotene, into two molecules of all-trans retinal, which then can be irreversibly oxidized into retinoic acid or reversibly reduced into retinol [5, 6]. Unlike BCMO1, BCO2 was demonstrated to catalyze asymmetrical cleavage of carotenoids at 9', 10' double bond, forming apocarotenoid, β-apo-10'-carotenal and β-ionone. BCO2 was found to have a much broader range of substrates, including β-carotene, lutein and zeaxanthin, compared with BCMO1, which majorly functions to catalyze the pro-vitamin A carotenoids [4, 7, 8].

Apart from its important enzyme activity in cleaving carotenoids, BCO2 was also

shown to mediate the development of inflammation [9] and macular degeneration [10] in humans. According to animal studies, BCO2 was shown to affect the development of anemia and apoptosis of red blood cells. The protein level of BCO2 was declined significantly in apoptotic red blood cells in mice. After the onset of hyperglycemia, BCO2 expression declined significantly in *db/db* mice. Increased intake of a high fat diet supplemented with carotenoids led to elevated expression of BCO2 protein , which may be associated with prevention of insulin resistance and fatty liver in obese C57BL/6J mice [11]. These observations suggest that BCO2 may play other roles than its enzymatic functions.

A recent study showed that BCO2 is located in the inner mitochondrial membrane [12], where facilitates the complexes of the electron transport chain/oxidative phosphorylation (ETC/OXPHOS) machinery. Mitochondria are considered as a power plant of the cell that generates most of the ATP to meet the cellular energy demand. In addition, mitochondria are also involved in numerous cellular processes including redox homeostasis, cellular signaling, development, growth, differentiation, and death. Thus, mitochondrial function is critical to cell survival and death [13-15]. A large number of studies clearly showed that disturbance of mitochondrial function is associated with a wide range of human diseases, including but not limited to obesity [16, 17], diabetes [18-20], aging, and neurological disorders [21, 22]. Mechanistic studies indicated that alterations in the ETC/OXPHOS [23, 24], the Krebs cycle [25], production of reactive oxygen species (ROS) [26], and overall mitochondrial membrane integrity [27] and mitochondrial stress [28] are major indicators of mitochondrial functions in health and diseases. Leakage of protons and/or electrons in the mitochondria results in enhanced

generation of ROS and decreased efficiency of ATP production, which in turn leads to cellular oxidative stress, inflammation, and initiation of obesity and diabetes [29, 30]. Yet, the impact of BCO2 on mitochondrial function, such as the mitochondrial complex activities, ETC efficiency, the Kreb's cycle, ROS generation, ATP production, and proton leak remains unknown. Liver is the major organ of carotenoid metabolism and storage in the body [31] and BCO2 was found to be significantly high in hepatocytes, which are essential for carotenoids metabolism [32, 33]. Thus, the role of BCO2 in mediation of hepatic mitochondrial functions needs to be investigated.

We recently generated a novel obesity model – the BCO2 knockout (KO) mouse. Under normal condition, the KO mice are not obese when fed a standard chow (10% kcal from fat), though they consume more food than the wild type genetic background (WT, 129S6/SvEv) mice. However, they are more obese than WT when fed a high fat diet (45% kcal from fat), indicating that BCO2 may be critical to obesity prevention (our unpublished data). Thus, the unique characteristics of this mouse model make it an appropriate model to investigate the mitochondrial function and/or diet-induced obesity.

The hypothesis of this study is that BCO2 plays an essential role in maintaining hepatic mitochondrial function by targeting the ETC. Complete ablation of BCO2 causes hepatic mitochondria to be more susceptible to cellular stress, more severe proton leak and subsequent damage. The following three specific objectives have been developed to test the hypothesis.

Objective 1: To determine how BCO2 mediates the mitochondrial function (proton leak, mitochondrial complex activities, and ATP production) in wild type and BCO2 knockout mice.

Objective 2: To profile BCO2 knockout-altered hepatic mitochondrial proteome in mice.

Objective 3: To characterize the impact of BCO2 on global status of cellular oxidative stress and lipid metabolism in mice (WT *versus* KO) via the global metabolomics approach.

CHAPTER II

REVIEW OF LITERATURE

BCO2 is not only an important enzyme in carotenoid metabolism, but also participates in other process, including inflammation [9], macular degeneration [10], development of anemia and fatty liver [11]. This review of the literature will include an overview of the carotenoid metabolism by BCMO1 and BCO2, effects of *BCO2* gene mutation, protein function of BCO2 in nutrient metabolism and cellular oxidative stress, and mitochondrial function and dysfunction.

Carotenoid Metabolism by BCMO1 and BCO2

Carotenoids are lipophilic pigments which contain 40 carbon atoms and can be synthesized in plants, bacteria and fungi. They are natural fat-soluble pigments, playing important role in animal and plant physiology. For example, β-carotene can be used as vitamin A precursor by animals [1]. Carotenoids are also considered as efficient scavengers of free radicals [2]. Over 600 known carotenoids have been classified into two groups: the xanthophyll, which contain oxygenated groups, and the carotenes, which are hydrocarbons without oxygenated groups and less polar than the xanthophyll [3, 4].

For carotenoid metabolism, the existence of carotenoid cleavage enzymes was discussed as early as 1965, however, the first cleavage enzyme VP14 was not identified until 1997 [34]. In animals, there are two carotenoids cleavage enzymes that have been identified. The first is BCMO1, which catalyzes the centric cleavage at 15, 15' double bond of carotenoids, forming two molecules of all-trans retinal, which then can be irreversibly oxidized into retinoic acid or reversibly reduced into retinol [5, 6] (**Fig. 1.**). The second enzyme is BCO2, which participates in eccentric cleavage of carotenoids at 9', 10' and/or 9, 10 double bond, forming apocarotenoid different from retinoid, including β-apo-10'-carotenal and β-ionone. Although BCMO1 functions as a major enzyme in vitamin A production, BCO2 is considered as an alternative pathway for forming vitamin A [7, 35]. BCMO1 and BCO2 are family members of nonheme iron oxygenase which are important in many physiological processes in animals and plants [36]. Beside BCMO1 and BCO2, the retinal pigment epithelium protein of 65 kDa (RPE65) was also included in this nonheme iron oxygenase family, which is crucial for vision [37, 38].

In recombinant human BCMO1 study, BCMO1 was found to cleave pro-vitamin A carotenoids, which contain at least one nonsubstituted β-ionone ring, such as α-carotene, β-carotene, or β-cryptoxanthin, but fails to catalyze the cleavage of non-pro-vitamin A carotenoids, such as zeaxanthin, lutein or lycopene [6]. BCMO1 was found to be present in the intestinal mucosa, where it facilitates the cleavage of majority of the carotenoids. BCMO1 was also presented in the classical steroidogenic cells, which are sensitive to vitamin A deficiency [39]. In this way, BCMO1 was proposed to be present in extra-intestinal tissues as a backup system for local synthesis of vitamin A when intake was decreased [8]. In mice, BCMO1 was found to play key roles in the retinoid

production [40]. In humans, a heterozygotic mutation in *BCMO1* gene led to the increased plasma levels of β-carotene and decreased plasma concentrations of retinoid [41].

The expression and activity of BCO2 has been verified in humans [39], cattle [42], sheep [43] and chicken [44]. BCO2 is highly expressed in hepatocytes, which are important for uptake and processing of retinol [32]. It has been shown that BCO2 cleaves β-carotene at the 9', 10' double bond producing β-ionone and β-apo-10'-carotenal [45]. BCO2 was expressed in *Escherichia coli*, which used carotenes and xanthophyll as substrates to produce β-ionone and β-apo-10'-carotenal [46]. Similar experiments show that lutein, β-cryptoxanthin and zeaxanthin are all specific substrates for BCO2. BCO2 was also found to cleave lutein at both 9', 10' and 9, 10 double bond, leading to the formation of β-ionone, β-apo-10'-carotenal and apo-10, 10'- carotenedialdehyde [47] (**Fig. 2.**). Interestingly, the production of β-apo-10'-carotenal was significantly affected by the concentration of Fe^{2+}. When the concentration of Fe^{2+} decreased from 78% to 67%, the formation of β-apo-10'-carotenal was significantly decreased, showing that iron was a necessary cofactor for this enzymatic cleavage [35]. In addition to β-carotene, BCO2 could also cleave the acyclic carotenoids, lycopene. However, only the 13-cis- and 5-cis-isomers of lycopene could be cleaved by BCO2. Recent research demonstrates that BCO2 displays much broader substrate specificity for carotenoids [12, 35, 48, 49].

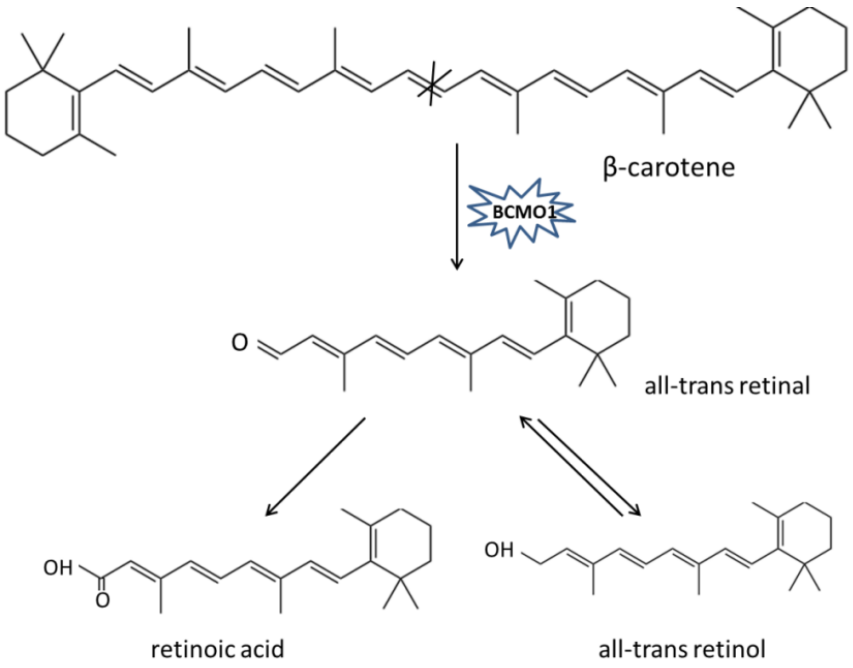


Fig. 1. BCMO1 Reaction Mechanism. In mammals, BCMO1 catalyzes carotenoids by cleaving double bond at position 15, 15' of carotenoids. BCMO1, β-carotene-15, 15'-monooxygenase.

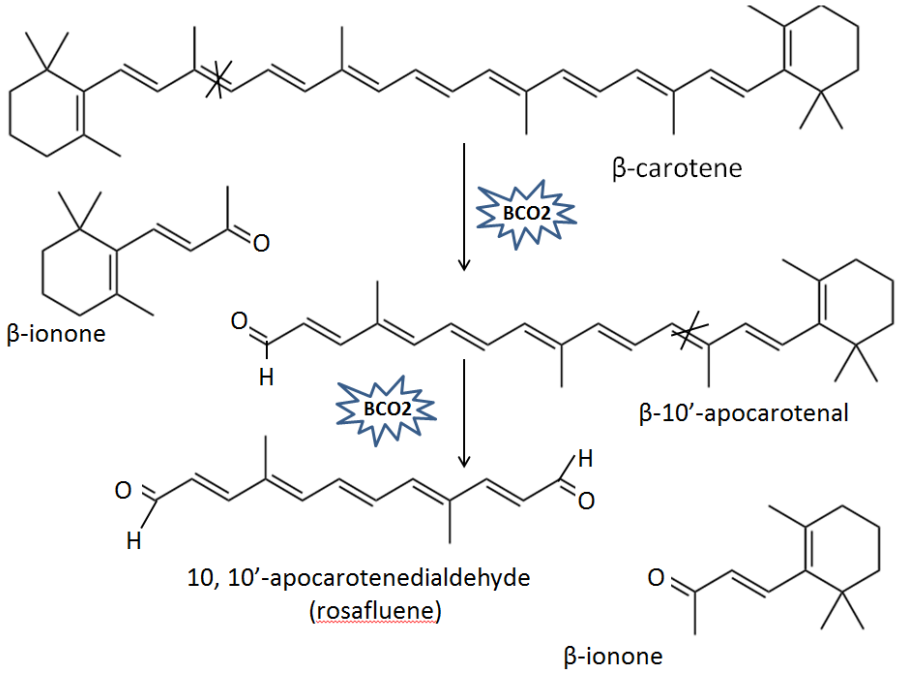


Fig. 2. BCO2 Reaction Mechanism. In mammals, BCO2, which is located in the inner mitochondrial membrane, catalyzes carotenoids by cleaving double bond at position 9, 10 and 9', 10' of carotenoids. BCO2, β,β-carotene-9', 10'-oxygenase 2.

The Effects of *BCO2* Gene Mutation

The deficiency of *BCO2* was found to be associated with carotenoids accumulating in the adipose tissues, such as subcutaneous adipose tissue [50], which leads to occurrence of yellow fat in sheep [51], cow [42] and chicken [44]. In the yellow fat experimental sheep, the *BCO2* coding region was sequenced and the nonsense mutation of *BCO2* was found in nucleotide position 196 (c.196C>T). This nonsense mutation introduced a stop codon in position 66 of amino acid, while the full-length protein BCO2 in sheep contains 575 amino acids. It was hypothesized that the mutant in *BCO2* gene forms a non-functional enzyme [52].

In cows, BCO2 was associated with the yellow color of the adipose tissue and milk, which was mainly caused by deposition of carotenoids [42]. Association analysis showed that there were significant differences among cattle with different *BCO2* genotypes in concentration of β-carotene and color of subcutaneous fat. Compared with genotypes of *BCO2* AA, GA and GG, the animals with AA had a higher concentration of β-carotene and more yellow fat than those animals with GG or GA genotype. The A allele which is in bovine *BCO2* exon 3 is a nonsense mutation. As the full length of BCO2 protein in cattle is 530 amino acids, the change in this allele results in a different polypeptide with presumably a lost in BCO2 enzyme function presumably [53]. The data showed that there is a strong link between this *BCO2* SNP and fat color in cattle.

IL-18, found in Kupffer cells and macrophages, is an important pro-inflammatory cytokine which pays key roles in innate and acquired immunities. It has been shown that the plasma concentration of IL-18 is associated with different diseases, including

atherosclerosis, cardiovascular diseases and type 2 diabetes [9, 54-56]. In humans, variants at the *BCO2* locus were associated with IL-18 levels, but not carotenoid levels in either plasma or macula [9]. A 2-stage genome-wide association study among women of Women's Genome Health Study (WGHS) and European ancestry from the Nurses' Health Study (NHS) was performed to test this association between *BCO2* and *IL-18*. In the stage of discovery, 7 SNPs located at the *IL18-BCO2* locus were significantly related with concentrations of IL-18. According to these combined analyses, SNPs rs2115763, rs7106524 and rs1834481 were found to show significant association between the *BCO2* and *IL-18* gene. SNP rs2115763 was found to be the strongest among these three SNPs. Further selection analysis showed that SNPs rs1834481 and rs2115763 were associated with IL-18 levels independently. Variation of 2.9% plasma levels of IL-18 could be explained together by these 2 SNPs. SNP rs2115763, which presents the strongest association, is located in intron 2 of the *BCO2* gene and also in the same location of the *IL18* gene [9]. However, the underlying mechanism between the IL-18 level and SNP rs2115763 in the BCO2 gene is unknown. SNP rs2115763 might regulate transcription of *IL18* gene directly, as the *BCO2* gene is upstream of the *IL18* gene. Another possibility is that SNP rs2115763 is an indicator of *IL18* gene functional mutation, affecting plasma levels of *IL-18* [54-56].

The Function of BCO2 Protein in Nutrition

Genetic studies in sheep, cows and chickens revealed that *BCO2* gene mutations could alter homeostasis of carotenoid metabolism. This was also confirmed in a mouse model. In accordance with the subcellular location of BCO2 (in the inner mitochondrial membrane), carotenoids were found to be accumulated in mitochondria and impair

respiration [12]. This process leads to cellular oxidative stress and alteration of signaling pathways related to cell proliferation and survival. Therefore, BCO2 functions as a key regulator of preventing the adverse effect caused by excess carotenoids. However, we are still at the early stage of understanding the importance of BCO2 in health and disease. The advanced knowledge of BCO2 function might help us to better understand the biochemical, developmental, and physiological roles of carotenoids.

Knock Down of BCO2 Causes Anemia and Apoptosis of Blood Cells

To further understand the importance of BCO2 in development, mRNA was used to knock down *BCO2* gene in zebrafish [57]. No defects in growth pattern or obvious malformations of organ systems, such as eyes and brain, were observed in BCO2 knock down (BCO2 -/-) morphants [46]. However, the red blood cells in BCO2 -/- morphants were no longer red and the blood filling rate of heart was also reduced in BCO2 -/- morphants compared with wild type groups. All these results showed that knock down of BCO2 caused anemia. Although the primitive erythropoiesis was not affected in BCO2 -/- morphants, the expression of a later erythrocyte differentiation marker, embryonic α-globin (hbae3), was increased. During the same time, fragmented nuclei were also presented in erythrocytes of BCO2 -/- morphants, indicating that occurrence of apoptosis of red blood cells [58, 59]. It was also reported that the differentiating blood cells were more sensitive to apoptosis when BCO2 was deficient. Thus, BCO2 is associated with the development of red blood cells [46].

BCO2 Is Related to Carotenoid-induced Oxidative Stress

Lobo GP and his colleagues showed that the protection of cells against apoptosis induced by carotenoid is strictly dependent on BCO2 enzymatic function [60]. After transfecting with murine *BCO2* gene, transfected cells could transiently express BCO2. Compared to control cells, the mitochondrial membrane was more polarized in transfected cells. Intrinsic apoptotic markers were assessed in these transfected cells after treatment with carotenoid. Activated caspase-3, which is a mediator of intrinsic apoptosis, was not changed in the transfected cells compared to control cells. Conversely, knock down of BCO2 in cells caused increased ROS production when cells were treated with carotenoids. More importantly, these BCO2 -/- cells showed initiation of apoptosis after incubation with carotenoid for 2 hours [60, 61]. Thus, BCO2 expression is associated with cellular oxidative stress (**Fig. 3.**).

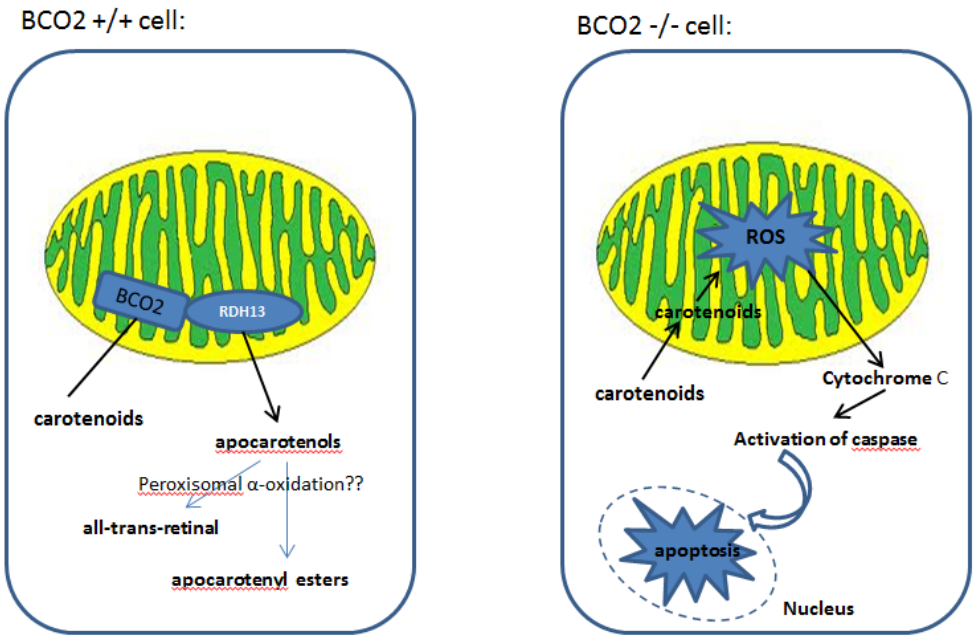


Fig. 3. The Role of Mitochondrial BCO2 in Cells. In cells that express endogenous BCO2, carotenoids can be degraded into apocarotenoids by BCO2. The apocarotenoids can then be metabolized by RDH13 into corresponding apocarotenols. These apocarotenols might then be metabolized by peroxisomal α-oxidation into all-trans-retinal or apocarotenyl esters. In cells without expression of BCO2, carotenoids could accumulate in the mitochondria. This kind of accumulation would interfere with respiration and produce ROS. These changes could cause cytochrome C release and then activate the caspase and eventually lead to cell apoptosis.

BCO2 Is Important in Prevention of Hepatic Steatosis

A high fat diet induces hepatic steatosis in mice through a possible mechanism of increasing ROS levels and suppressing mitophagy and mitochondrial biogenesis. Hepatic concentration of zeaxanthin and lutein and expression of BCO2 in hepatic mitochondria were lowered in mice fed with a high fat diet. These results suggested that BCO2 might be a stress responsive gene in mice [33]. BCO2 knockout mice also showed lipid accumulation in liver [46]. However, elevated expression of BCO2 attenuated the hepatic steatosis. All these results indicated that BCO2 protein is important in prevention of hepatic steatosis. Knockout of BCO2 might cause mitochondrial dysfunction in the liver and increased hepatic cell susceptibility to oxidative stress [62-65].

Mitochondrial Function and Dysfunction

Mitochondria are the major sites of ATP production in the cells by aerobic respiration, which tightly couple oxygen consumptions with ATP production. However, the mitochondria have many other functions aside from the production of ATP, including the generation of important metabolites necessary for fatty acid synthesis, steroid synthesis, ketogenesis, amino acid metabolism and the urea cycle, production of cellular ROS, calcium signaling (including calcium-evoked apoptosis), regulation of membrane potential and a variety of cell signaling pathways, including key roles as effectors of apoptosis and cell death. Thus, the integrity of mitochondria is critical for cell function and survival [13-15, 66].

Recent studies indicate that mitochondrial dysfunction has been associated with a number of diseases, such as obesity [67], insulin resistance [18, 19] and diabetes [16, 20].

There is compelling evidence suggesting that mitochondrial dysfunction, mediated by changes in mitochondrial gene expression, mitochondrial turnover and morphology, contributes to insulin resistance in diabetes [19, 20, 68]. However, some other publications showed that insulin resistance also caused mitochondrial dysfunction. The transcription of mitochondrial genes such as mitofusin (Mfn), and the subunits I and IV of the cytochrome c oxidase (COX I and COX IV) in cultured myoblasts were regulated by insulin administration [69, 70]. Biogenesis of mitochondria is linked to insulin signaling via dFOXO-mediated repression on nutrient responsive genes: including PGC1 homologue [PPAR (peroxisome-proliferator-activated receptor) γ coactivator-1], a transcriptional co-activator implicated in controlling mitochondrial gene expression in mammals [71]. In vivo, insulin administration to non-diabetic insulin sensitive humans caused an increase in the mitochondrial ATP production rate, an effect that was not observed in diabetic patients [72]. Synthesis of mitochondrial protein was also found to be impaired in diabetic subjects [73]. Therefore, mitochondrial function is tightly associated with the development of diabetes.

Obesity and overweight are epidemic health problems in the United States. Over-nutrition is one of the factors attributed to the development of obesity and overweight. Energy oversupply, particularly of fatty acids, impacts mitochondrial activity and contributes to the development of mitochondrial dysfunction [67, 74, 75]. The rate of mitochondrial ATP synthesis was rapidly decreased by switching animals to a high-fat diet for a few days [75]. The genes coding proteins in mitochondrial biogenesis and oxidative phosphorylation were down-regulated due the high-fat feeding [74]. Furthermore, increased levels of fatty acid, as presented in obesity and overweight,

resulted in the production and accumulation of ceramides and diacylglycerol, which also contributed to suppression of the phosphoinositide 3-kinase signaling pathway, thus increasing insulin resistance [76]. In this way, mitochondrial dysfunction is one major manifestation in obesity and overweight subjects.

In this literature review, the importance of BCO2 in carotenoid metabolism was discussed. Genetic studies in sheep, cows and chickens showed that *BCO2* gene mutations could alter homeostasis of carotenoid. Aside from the enzymatic roles in carotenoid metabolism, BCO2 has lots of other functions, including inflammatory response, protection against oxidative stress, apoptosis of red blood cells and hepatic steatosis. One recent publication pointed out that BCO2 was located in the inner mitochondrial membrane, indicating that BCO2 is essential for maintaining mitochondrial membrane potential. However, we are still at the early stage of understanding importance of BCO2 in health and disease, especially the impacts on mitochondria function. Until now, there are limited studies conducted about the function of BCO2 on ETC/OXPHOS and mitochondrial function. As mitochondrial dysfunction plays a key role in the development of obesity and diabetes, the possible role of BCO2 in mediation of hepatic mitochondrial functions needs to be investigated. Therefore the purpose of this study was to determine how BCO2 mediates the hepatic mitochondrial function via targeting at ETC/OXPHOS.

CHAPTER III

METHODOLOGY

Animals and Animal Care

The breeding colonies of both strains, the genetic background wild type 129S6 (WT) and BCO2 knockout (KO), were maintained in the Animal Resources Facility at Oklahoma State University. Male wild type and knockout mice at 6 weeks of age were used in this study. Mice were group-housed (2-3 mice/cage) in a controlled environment with a 12-h light/dark cycle. Animals had free access to water and food throughout the study, according to the Institutional Animal Care and Use Committee (IACUC) protocol. Mice were fed a regular chow diet (CD, 10 % kcal from fat, catalog # D12450B, purchased from the Research Diets, Inc., New Brunswick, NJ). Liver tissues were collected for laboratory analysis.

Mitochondrial Proteome

Hepatic mitochondria were isolated using a Qproteome mitochondrial isolation kit (Qiagen, CA). Partial purified mitochondrial samples (24 ug/sample) were subjected to LC-MS/MS in the Proteomics Core Facility at Oklahoma State University. The

experiments had 3 biological replicates, and 4 technical replicates.

Gene Ontology Annotation

The DAVID 6.7 Bioinformatics tool (http://david.abcc.ncifcrf.gov/) was used for gene functional categorization and pathway analysis. The above selected proteins from mitochondrial proteomic data, which were significantly changed, were loaded into DAVID. The DAVID provided gene-gene ontology (GO) term enrichment analysis and protein functional annotation to highlight the most relevant GO terms based on these selected proteins [77, 78].

Ingenuity Pathway Analysis (IPA)

The selected protein functional analysis was analyzed by IPA. IPA provided canonical pathways, upstream regulator analysis, and downstream effects analysis based on these selected proteins [79, 80].

Cytoscape Analysis

The *p*-values of selected proteins from mitochondrial proteome were mapped into publicly available protein-protein interaction network using Cytoscape [81, 82]. Cytoscape provided a global view of potentially relevant interacting partners of proteins whose abundance were changed in knockout mice. Possible functional links from GeneMANIA among significant proteins using computational prediction were searched based on several criteria including co-expression of genes and co-localization of proteins [83, 84].

Electron Microscopy

The fresh liver was extracted and immersed into a glutaraldehyde fixing solution (pH 7.0) and sliced into smaller and thinner pieces (less than 1mm). The volume of fixing solution was more than10 times the volume of the tissue. Samples were washed with washing buffer (0.1 M sodium cacodylate, 3 mM calcium chloride, pH 7.4) on ice for three times and then fixed with 1% osmium tetroxide buffer for 1 hour. The samples were then washed with distilled water for 3 times on ice and dehydrated through graded ethanol solutions (50%, 70%, 90%, 95% and three times in 100% ethanol). The samples were then washed with distilled water for 3 times on ice. Samples were then immersed into propylene oxide overnight. After infiltration, the samples were embedded in 100% embedding medium at 60 °C. The samples were sectioned into thinner layers using a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) and stained in 2% aqueous uranyl acetate for 30 minutes. After the first staining, the samples were rinsed with water for three times on ice. Lastly, the samples were stained again in Reynold's lead citrate and rinsed with CO_2 free water for 3 times on ice. The prepared samples were observed under electron microscope at 80 kV [85].

Western Blot Analysis

To verify LC-MS data, knockout and wild type liver were subjected to western blot analysis. For this purpose, liver samples were lysed with ice-cold whole cell lysis buffer (20 mM tris, 0.5 mM EGTA, 0.5 mM EDTA,0.5% triton X-100 and 1% protease and phosphatase inhibitors) and then homogenized (Qiagen tissuerupter, CA) for 10s at the lowest speed. After homogenization, the sample suspension were sonicated and

centrifuged at 8000g for 10 minutes (at 4 °C). The supernatant were aliquoted and stored at -80 °C. Protein samples were denatured at 80 °C water for 3 minutes before loading into SDS-PAGE gels. Based on protein concentrations, tested by bicinchoninic acid assay (BCA assay, Pierce, Rockford, IL), only 50 ug proteins were loaded into each well. After separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes and probed with corresponding antibodies. Significantly changed proteins in proteomic data, which played important roles in cell survival, apoptosis, inflammation and regulating gene transcription, were tested. The following antibodies were used: NACHT, LRR and PYD domains-containing protein 3 (NALP3), caspase-1, protein kinase-like endoplasmic reticulum kinase (PERK), cytochrome c oxidase subunit IV (COX IV), β-actin, β-catenin, interleukin-18 (IL-18), superoxide dismutase (SOD), C/EBP-homologous protein (CHOP) , peroxisome proliferator-activated receptor alpha (PPARα), cleaved poly ADP-ribose polymerase (cleaved PARP), NAD(P) transhydrogenase (NNT) and mammalian target of rapamycin (mTOR). However, in the results section, only SOD, mTOR, NNT, AMPKα and PPARα were presented. Protein expressions were normalized using loading control, which is β-actin in the present study.

Mitochondrial Isolation

Fresh liver from knockout and wild type mice were used to extract mitochondria for mitochondrial function analysis. Liver tissues (60 mg) were extracted and minced in 0.5 mL mitochondrial isolation buffer (MSHE: 210 mM mannitol, 70 mM sucrose, 5 Mm HEPES,I mM EGTA and 0.5% (w/v) fatty acid-free BSA, pH 7.2) and followed by homogenization (Qiagen tissuerupter, CA) for 10s at the lowest speed. The homogenate were centrifuged at 600g for 5 minutes at 4 °C and the supernatant were transferred into

new tubes and centrifuged again at 5000g for 5 minutes at 4 °C. After removing all the supernatant, the final pellet were resuspended with 1 mL mitochondrial assay solution (MAS 1X: 70 mM sucrose, 220 mM mannitol, 5 mM $MgCl_2$, 2 mM HEPES, 10 mM KH_2PO_4, 1 mM EGTA, 0.2% (w/v) fatty acid-free BSA) at 4 °C. Mitochondria from each mouse (3 wild type mice, 3 BCO2 knockout mice) were isolated separately. Total protein concentrations (ug/mL) were determined using the bicinchoninic acid assay (BCA assay, Pierce, Rockford, IL).

"Coupling Assay" Respiration Analysis Using the Seahorse *XF^e 96* Flux Analyzer

The isolated mitochondria were used to run "coupling assay" respiration assay, which was designed to test mitochondrial ETC efficiency and proton leak. All the steps were conducted on ice unless otherwise stated. Firstly, isolated mitochondria were diluted into lower concentration using 1X MAS after quantitation of protein concentration by BCA assay. The diluted mitochondria (90 uL) were loaded into individual wells of a *XF^e 96* cell culture microplate. The microplate was transferred to a centrifuge with a swinging bucket microplate adaptor, and centrifuged at 2000g for 10 minutes (at 4 °C). Prewarmed (37 °C) 1X MAS (supplemented with 10 mM pyruvate, 10 mM malate, and 10 mM succinate) were added into each well carefully. The mitochondria were viewed briefly under microscope to ensure consistent adherence to the well. The plate was then transferred to the Extracellular Flux *XF^e* 96 Analyzer (Seahorse Bioscience Inc., MA) and the experiment was initiated. Subsequent oxygen consumption rate (OCR) analysis of isolated hepatic mitochondria were conducted according to the "coupling assay" protocol (mix/measure time-course) and drug injections. Injections were as follows: port A, 20 uL of 40 mM ADP (4 mM, final); port B, 22 uL of 15 uM oligomycin (1.5 uM, final); port C,

24 uL of 40 uM FCCP (4 uM, final); port D, 26 uL of 40 uM antimycin A (4 uM, final). The final volume of each well was 272 uL. The running protocol was described in **Table 1**.

"Electron Flow" Respiration Analysis Using the Seahorse *XF^e96* Flux Analyzer

The above diluted mitochondria were used in electron flow respiration analysis, designed to examine sequential electron flow through different complexes of mitochondrial ETC, which can identify the mechanism of mitochondrial dysfunction together with the coupling assay. The diluted mitochondria (90 uL) were loaded into individual wells of a *XF^e* 96 cell culture microplate. The microplate was transferred to a centrifuge with a swinging bucket microplate adaptor, and centrifuged at 2000g for 10 minutes (at 4 ^{o}C). Prewarmed (37 ^{o}C) 1X MAS (supplemented with 10 mM pyruvate, 10 mM malate, and 4 uM FCCP) was added into each well carefully. The mitochondria were viewed briefly under microscope to ensure consistent adherence to the well. The plate was then transferred to the Extracellular Flux *XF^e* 96 Analyzer (Seahorse Bioscience Inc., MA) and the experiment was initiated. Subsequent oxygen consumption rate (OCR) analysis of isolated hepatic mitochondria was conducted according to the given "electron flow" protocol (mix/measure time-course) and drug injections. The injections were as follows: port A, 20 uL of 20 uM rotenone (2 uM, final); port B, 22 uL of 100 mM succinate (1.5 uM, final); port C, 24 uL of 40 uM antimycin A (4 uM, final); port D, 26 uL of 100 mM ascorbate/ 1 mM TMP (final 10 mM and 100 uM, respectively). The final volume of each well was 272 uL. The running protocol was described in **Table 1**.

Start Protocol		
Command	**Time(min.)**	**port**
Calibrate	**20**	
Equilibrate	**12**	
mix	**1**	
wait	**1**	
measure	**3**	
mix	**1**	**A**
measure	**3**	
mix	**1**	**B**
wait	**1**	
measure	**3**	
mix	**1**	**C**
measure	**3**	
Mix	**1**	**D**
measure	**3**	
	End Protocol	

Table 1. Seahorse *XF*^{e} 96 Extracellular Flux Analyzer Running Protocol. "Coupling Assay" and "Electron Flow" respiration analysis used same running protocol. The difference between the two analyses is the different injections. In "Coupling Assay", the injection A is 40 mM ADP, injection B is 15 uM Oligomycine, injection C is 40 uM FCCP and injection D is 40 uM antimycine A. In" Electron Flow", the injection A is 20 uM rotenone, injection B is 100 mM succinate, injection C is 40 uM antimycine A and injection D is 100 mM /1 mM ascorbate/TMP.

Global metabolomics profiling

To examine the functional impact of BCO2 on global metabolic changes in the liver of mice fed with chow diet, a global metabolomics profiling assay was conducted by the Metabolon, Inc (Durham, NC), a pioneer and industry leader in metabolomics. In brief, the metabolites of liver samples (~50 mg liver tissues) were identified by ultrahigh performance liquid chromatography-tandem mass spectroscopy and gas chromatography-mass spectroscopy. Multiple bioinformatic analysis methods were used, including student's t-test, three-way ANOVA, Hotelling's T2 test, random forest, hierarchical clustering, and principal components analysis. Two types of statistical analysis were performed to analyze the collected data: (1) significance tests and (2) classification analysis.

Statistical Analyses

The proteomic data were analyzed using student's t-test. First, the fold changes were calculated by dividing KO data with WT data. Second, significant levels were calculated using student's t-test. The proteins with fold changes over 1.1 or less than 0.9 were selected among the significantly changes proteins. The selected proteins were analyzed by different bioinformatics softwares, including ontology annotation (DAVID 6.7), Ingenuity Pathway Analysis, Cytoscape and GeneMANIA.

The results from respiration assay were analyzed by the *XF*^{e} wave software (Seahorse Bioscience Inc., MA), and displayed as oxygen consumption rates (pMoles/min/well) *versus* time. All the data were presented as the mean of 3-6 replicated wells ± SD.

The final mean of respiration assay were normalized with protein concentration, which were tested using BCA assay. The normalized respiration data were used to compare the variables between wild type and knockout groups by student's t-test. Significance was set at $p < 0.05$, 0.01 and/or 0.001.

CHAPTER IV

RESULTS

Ablation of BCO2 Altered Hepatic Mitochondrial Proteomics in Mice

To gain how hepatic mitochondrial changes in BCO2-knockout mice, the selected proteins and pathways were analyzed using bioinformatics software. The hepatic mitochondria were isolated independent from three biological replicates (3 knockout and 3 wild type) in both mouse strains and then proteins were analyzed by LC-MS/MS. DAVID Bioinformatics was used to extract the pathways that were up- or down-regulated in BCO2 knockout compared with that of wild type samples [78].

To identify proteins that have significant changes in BCO2 knock out hepatic mitochondria, the cutoff of fold change was set as > 1.1 or < 0.9 for all three biological replicates and a two-tailed student's t-test with the *p* value cutoff of 0.05. The results were summarized in **Table 2**. Only 34 mitochondrial proteins were found to have significant changes with 13 proteins up-regulated and 21 proteins down-regulated in the hepatic mitochondria of BCO2 knockout *versus* wild type. The up-regulated proteins included enoyl-CoA delta isomerase 1(ECT1), amino methyltransferase (AMT), ornithine aminotransferase (OAT), citrate synthase (CS), probable 2-oxoglutarate dehydrogenase

dehydrogenase E1 component (DHKTD1), sulfite oxidase (SUOX), NNT, CYP1A2, CYP2D9, CYP2C40, CYP7B1, aldehyde dehydrogenase X (ALDH1B1), epoxide hydrolase (EPHX1) and persulfide dioxygenase (ETHE1). CYP1A2, CYP2D9, CYP2C40 and CYP7B1 are important mitochondrial proteins in retinoid metabolism. The elevated levels of these retinoid metabolism-associated proteins suggested that the vitamin A metabolism was elevated in BCO2 knockout liver compared to wild type. The level of NNT was increased dramatically, indicating increased ROS production in mitochondrial matrix.

The down-regulated proteins included probable phospholipid-transporting ATPase 11C (ATP11C), hydroxyacyl-coenzyme A dehydrogenase (HADH), glycine dehydrogenase (GLDC), isoform 2 of Mitochondrial peptide methionine sulfoxide reductase (MSRA), glutaminase (GLS2), sarcosine dehydrogenase (SARDH), glycine N-acyltransferase-like protein (GLYATL), succinyl-CoA ligase subunit beta (SUCLG2), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (NDUFA10), succinate dehydrogenase flavoprotein subunit (SDHA), mitochondrial import inner membrane translocase subunit (TIM50), voltage-dependent anion-selective channel protein 3 (VDAC3), isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1 (VDAC1), et al. In summary, the mitochondrial transporter proteins such as TIM50, VDAC1 and VDAC3 were significantly down-regulated in BCO2 knockout liver. Five of the down-regulated proteins were involved in the amino acid metabolism. The protein level of NDUFA10, subunit of mitochondrial complex I, and SDHA, subunit of mitochondrial complex II, were significantly lower in BCO2 knockout liver. However, it was difficult to make any conclusion based only on the listed proteins, as each group

contained both up-regulated and down-regulated proteins. **Table 2.** only show a list of pathways that were altered in the BCO2 knockout liver.

Regarding the use of DAVID Bioinformatics for pathways that were up-regulated or down-regulated, fatty acid oxidation, TCA cycle, amino acid metabolism and oxidative phosphorylation were affected by BCO2 knockout. Within the KEGG pathway, oxidative phosphorylation pathway was also disrupted by BCO2 knockout (**Fig. 4**).

The selected proteins were also analyzed by IPA. The canonical pathway analysis indicates mitochondrial dysfunction and oxidative phosphorylation were clearly affected in BCO2 knockout liver (**Fig. 5A**). The protein-protein interactions were also analyzed by IPA. In ETC/oxidative phosphorylation, the expression of mitochondrial complex I, complex III and complex IV were down-regulated and cytochrome C is up-regulated (**Fig. 5B**).

Gene symbol	Protein name	Ratio(KO/WT)	p-values
Fatty Acid Metabolism			
ATP11C	Probable phospholipid-transporting ATPase 11C	0.72973	0.044363
HADH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0.873362	0.018499
ECI1	Enoyl-CoA delta isomerase 1, mitochondrial	1.589041	0.014808
Amino Acid Metabolism			
GLDC	Glycine dehydrogenase (decarboxylating), mitochondrial	0.454545	0.029663
MSRA	Isoform 2 of Mitochondrial peptide methionine sulfoxide reductase	0.5125	0.02286
GLS2	Glutaminase liver isoform, mitochondrial	0.717557	0.027326
SARDH	sarcosine dehydrogenase, mitochondrial	0.831268	0.020334
GLYATL	Glycine N-acyltransferase-like protein	0.84878	0.016245
AMT	Aminomethyltransferase, mitochondrial	1.431818	0.039742
OAT	Ornithine aminotransferase, mitochondrial	1.689655	0.028714
TCA Cycle			
SUCLG2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	0.809231	0.04195
CS	Citrate synthase	1.237179	0.013596
DHTKD1	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial	2.870466	0.001524
ETC			
NDUFA10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	0.835714	0.053906
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0.874648	0.025268
SUOX	Sulfite oxidase, mitochondrial	1.298039	0.002759
NNT	NAD(P) transhydrogenase, mitochondrial	87.67/0	0.001097
Mitochondria Protein Translation			
AK4	Adenylate kinase 4, mitochondrial	0.5	0.033908
RPL7	ribosomal protein L7	0.715	0.003673
Vit A Metabolism			
UGT1A5	MCG14318, isoform CRA_h	0.706	0.022184
CYP1A2	CYP1A2 cytochrome P450, family 1, subfamily A, polypeptide 2	1.148515	0.029731
CYP2D9	cytochrome P450, family 2, subfamily d, polypeptide 9	1.177083	0.01356
CYP2C40	Cytochrome P450 2C40	3.0374	0.039512
CYP7B1	25-hydroxycholesterol 7-alpha-hydroxylase	12.625	0.038268
Protease			
CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial	0.73913	0.015268

Transporter Protein			
TIMM50	Mitochondrial import inner membrane translocase subunit TIM50	0.597222	0.031718
VDAC3	Voltage-dependent anion-selective channel protein 3	0.786802	0.001696
VDAC1	Isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1	0.910853	0.012976
Misc.			
HYOU1	Hypoxia up-regulated protein 1 protein folding HSP70 family	0.840816	0.049346
CES2A	Pyrethroid hydrolase Ces2a	0.916442	0.036412
CPS1	Carbamoyl Phosphate Synthetase I	0.938317	0.003359
ALDH1B1	Aldehyde dehydrogenase X, mitochondrial	1.109524	0.023704
EPHX1	Epoxide hydrolase 1	1.133047	0.05008
ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	1.27907	0.002307

Table 2. Functional Categorization of Significantly Different Proteins. Isolated mitochondria independently isolated from each mouse in both groups (BCO2 knockout *versus* wild type, 3 biological replicates of each group) were identified by Spectrum counting (LC-MS/MS). The cutoff of ratio (KO/WT) was > 1.1 or < 0.9. The significant level set as $p < 0.05$.

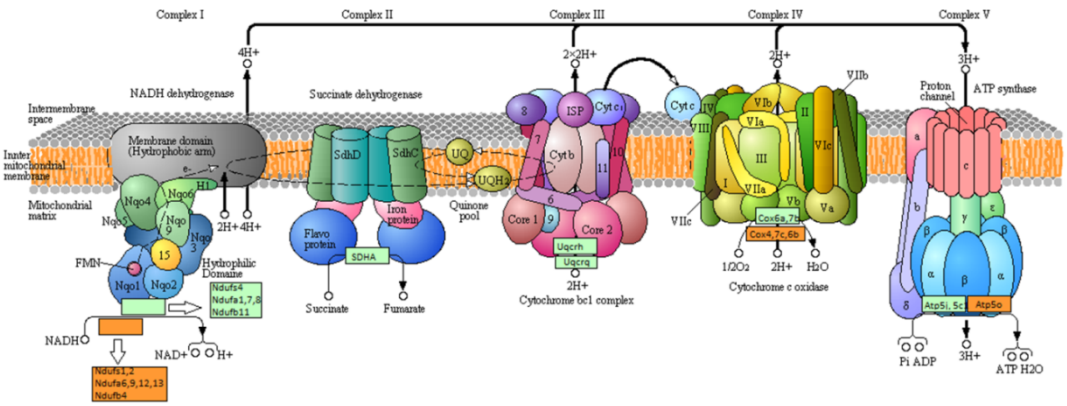


Fig. 4. KEGG Pathway for ETC in BCO2 Knockout Mice. The DAVID bioinformatics software was used to further analyze individual proteins that were up- or down-regulated in BCO2 KO mice as determined by proteomics. The proteins in green were consistently decreased. The proteins in yellow were consistently increased. Fig. 4. was downloaded from DAVID *(http://david.abcc.ncifcrf.gov/)*.

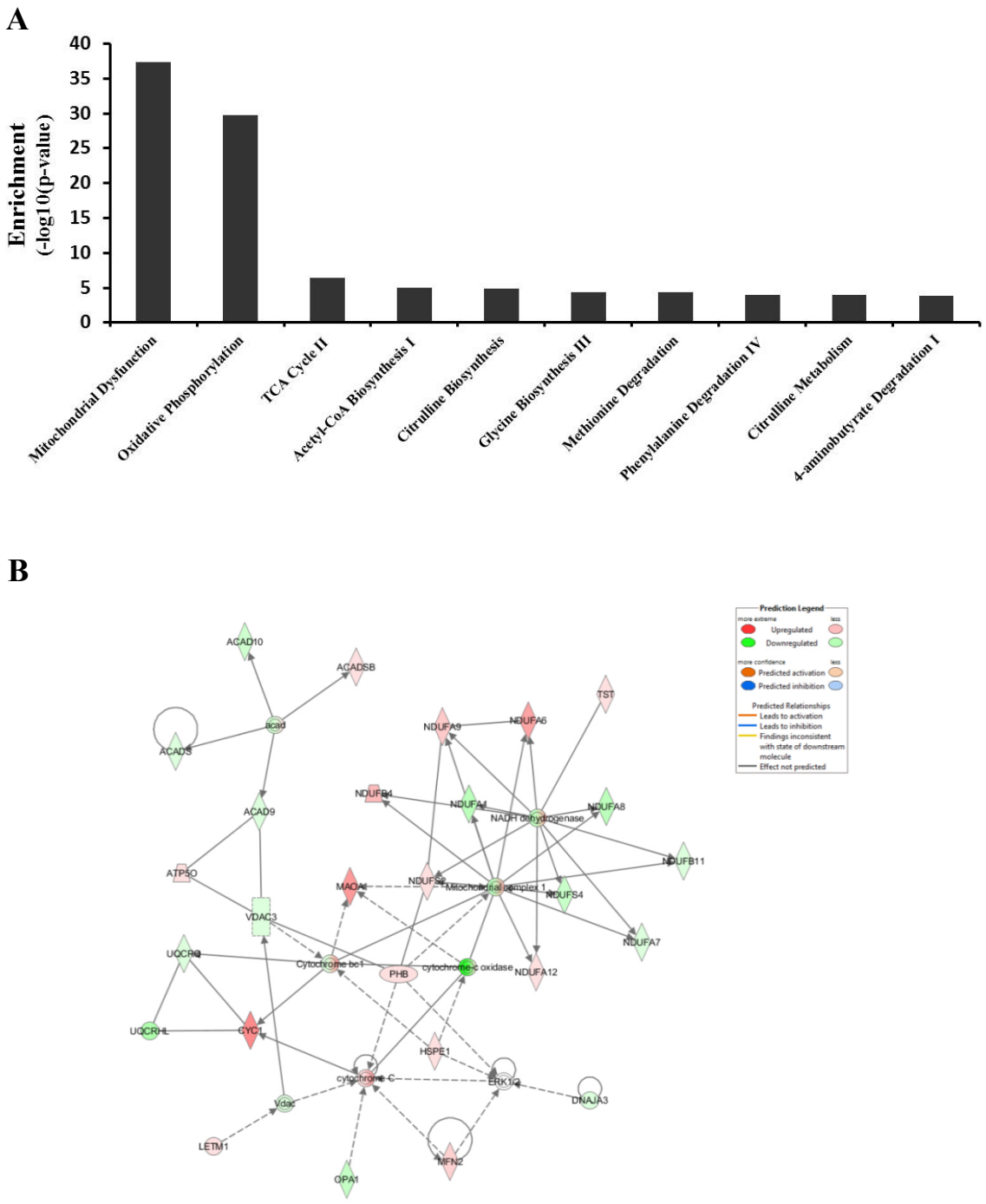
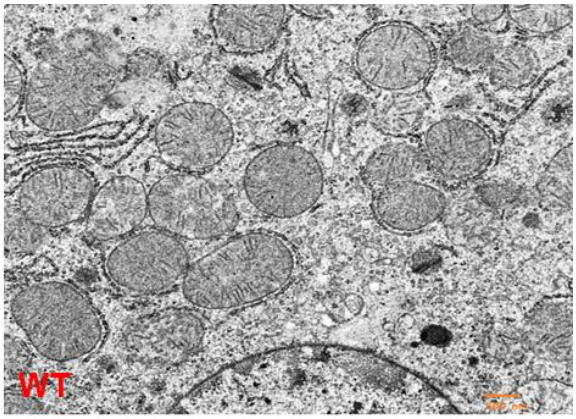


Fig. 5. BCO2 Knockout-altered Hepatic Mitochondrial Proteome in Mice. The list of proteins identified from LC-MS/MS was analyzed using the ingenuity pathway analysis (IPA). *A,* Canonical pathway analysis. The enrichment of different canonical pathways (-log10 p-value), which are larger than cutoff (4). *B.* Protein-protein interactions were analyzed using ingenuity pathway analysis (IPA). In the ETC/oxidative phosphorylation pathway, complex I, complex III and complex IV were down-regulated. Cytochrome C was up-regulated.

BCO2 Knockout Caused Structurally Alterations in Hepatic Mitochondria

BCO2 knockout-induced structural changes in hepatic mitochondria were analyzed by electron microscopy. The most obvious difference between the mitochondria from BCO2 knockout liver and their counterparts from wild type liver is the increased mitochondrial numbers and changed mitochondrial structures. Under same scale bar, the number of mitochondria was greater and the size of mitochondria was smaller in BCO2 knockout mice related to wild type mice. Increased glycogen granules and lipid droplets were also observed in BCO2 knockout hepatic mitochondria (**Fig. 6.**). As shown in **Fig. 6**, there were more black particles, which were considered as glycogen granules, in BCO2 knockout liver compared with wild type, indicating that glycogen synthase was activated or glycogen usage was inhibited when expression of BCO2 was suppressed. In addition to the above changes, the cell organelles in BCO2 knockout hepatocytes were more disorganized compared to wild type.

A



B

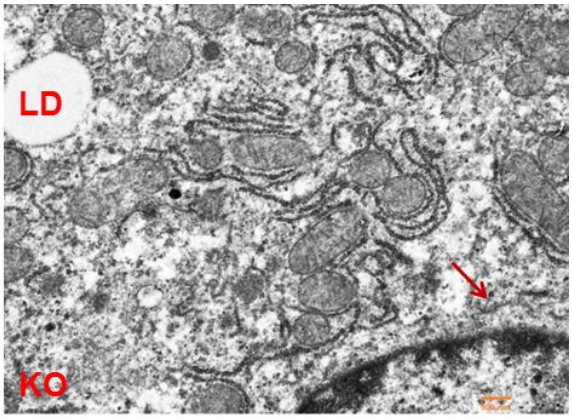
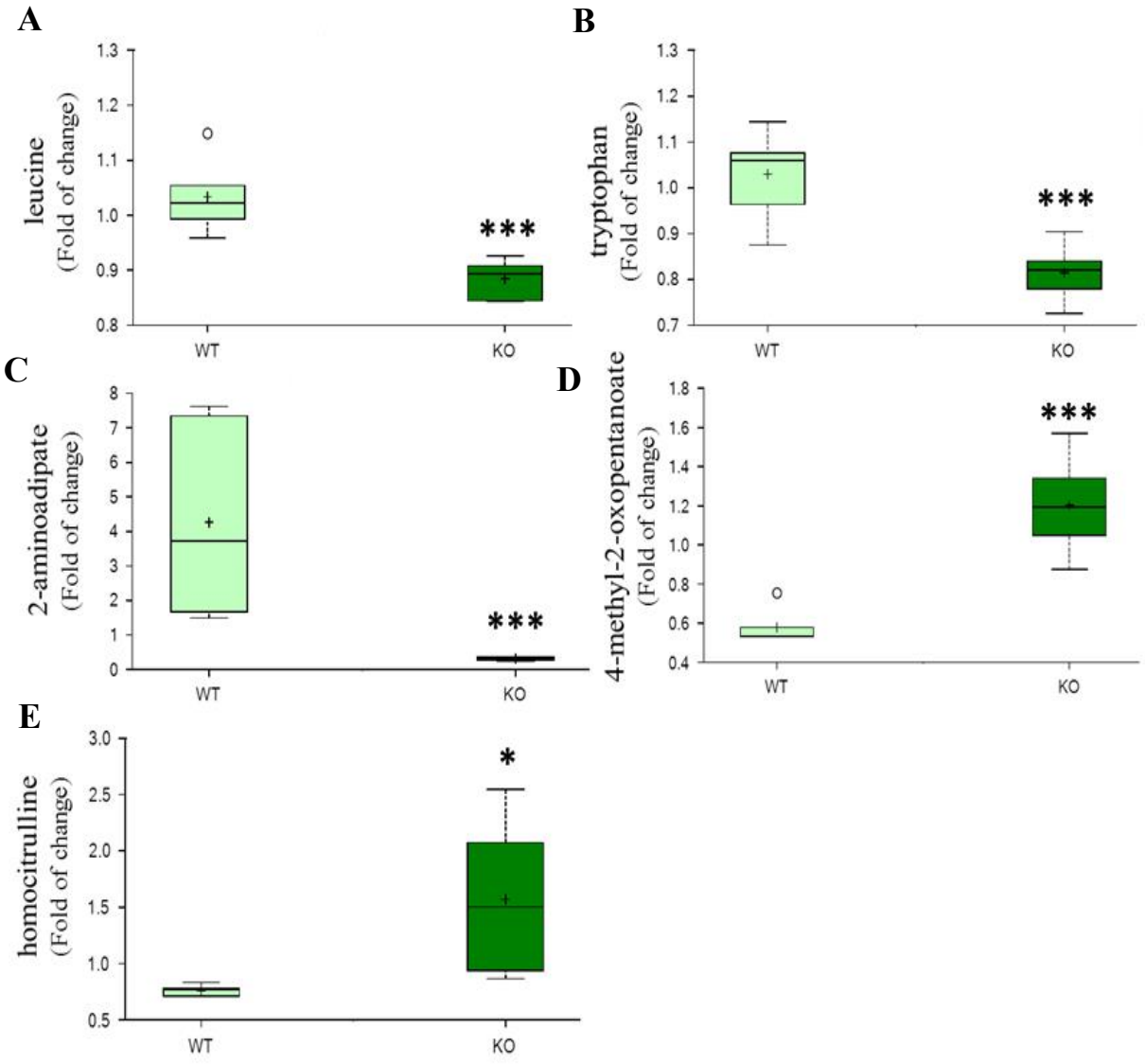


Fig. 6. BCO2 Impacts on Hepatic Mitochondria Structurally. Representative EM images for the wild type (WT) and BCO2 knockout (KO) liver. The morphology of hepatic mitochondria was changed in knockout mice compared to wild type. Increased glycogen granules (arrowed) and lipid droplets (LD) were also observed. *A,* typical mitochondria in wild type liver. *B,* typical mitochondria in knockout liver. Scale bar, 500 nm.

Markers of Mitochondrial Metabolism Were Altered in BCO2 Knockout Liver

As shown in proteomic data, there were broad signs of disrupted or at least altered mitochondrial function in the livers of BCO2 knockout mice. To verify these mitochondrial disruptions or alterations, a global metabolomics profiling assay was performed using fresh liver tissues from both 6-week BCO2 knockout mice and age-matched wild type mice. The levels of leucine and tryptophan were significantly decreased in BCO2 knockout mice compared with wild type mice (**Fig. 7A, B**). Similar results of their intermediates, such as 2-aminoadipate derived from lysine or tryptophan degradation, were found in BCO2 knockout mice (**Fig. 7C**). The disposal of leucine, tryptophan and their intermediates are dependent on the mitochondrial activity [86, 87]. On the other hand, 4-methyl-2-oxopentanoate, the deamination product of leucine, was significantly elevated in BCO2 knockout mice (**Fig. 7D**). Homocitrulline, which is formed when lysine is substituted for ornithine in the mitochondrial reaction catalyzed by ornithine transcarbamylase (OTC) [88], was also significantly increased in BCO2 knockout mice (**Fig. 7E**). These changes suggested that the oxidation of amino acids, such as leucine, tryptophan, and lysine, which are dependent on mitochondria, was restricted in BCO2 knockout mice. Based on the proteomic data, significantly changed protein related to mitochondrial metabolism, were verified by immunoblotting analysis. Mammalian/mechanistic target of rapamycin 1 (mTOR), an important indicator of mitochondrial autophagy [89, 90], was significantly increased in BCO2 knockout mice compared to wild type mice (**Fig. 7F, G**). Above all, markers of mitochondrial metabolism were altered in BCO knockout liver.


A
leucine
(Fold of change)
1.3
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0.8
***
WT
KO
B
tryptophan
(Fold of change)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
***
WT
KO
C
2-aminoadipate
(Fold of change)
8
7
6
5
4
3
2
1
0
***
WT
KO
D
4-methyl-2-oxopentanoate
(Fold of change)
1.8
1.6
1.4
1.2
1.0
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0.4
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WT
KO
E
homocitrulline
(Fold of change)
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WT
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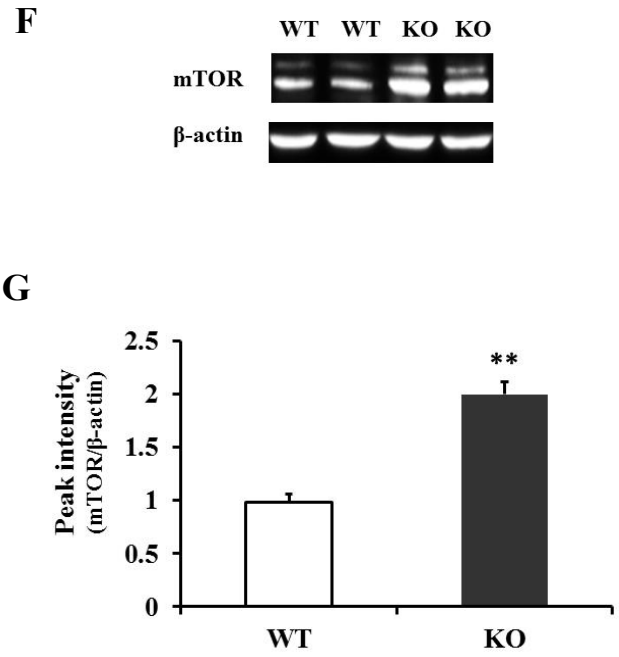


Fig. 7. Markers of Mitochondrial Metabolism Were Altered in BCO2 Knockout Liver. The global metabolomics profiling assay was performed using fresh liver from both 6-week BCO2 knockout mice and age matched wild type mice (n=6 biological replicates). *A,* leucine. *B,* tryptophan. *C,* 2-aminoadipate. *D,* 4-methyl-2-oxopentanoate. *E,* homocitrulline. *F,* increased expression of mTOR (lane 3, 4) in BCO2-knockout mice liver compared with wild-type mice (lane 1, 2). *G,* quantification of mTOR western blot. n=6 biological replicates. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Mitochondrial ATP Generation, Oxygen Consumption Rate (OCR) and Complex Activity Were Elevated with BCO2 Knockout

To determine whether or not BCO2 knockout induces a significant difference in mitochondrial metabolic capacity, functional analyses of isolated hepatic mitochondria from both 6-week BCO2 knockout mice and age-matched wild-type mice were performed. First, oxygen consumption measurements using XF^e 96 extracellular flux analyzer to assess the mitochondrial respiratory capacity among the two different groups were conducted. According to one independent experiment with 12 technical replicates for each group, a significant increase in basal respiration capacity in knockout mice compared with wild type mice was observed on both activation of complex I and complex II using malate, pyruvate and succinate as substrates (**Fig. 8A**). Based on the experimental design, a significant increase in the proton leak was found in the knockout mice compared with wild type mice (**Fig. 8B**). After quantification, the maximal ADP production was significantly increased in the knockout mice relative to wild type mice under malate/pyruvate/succinate stimulus for activation of both complex I and complex II driven respiration (**Fig. 8C**). As the "electron flow" experiments were performed using 3 biological replicates, the capacity of complex II was markedly increased in knockout mice as compared with wild type mice (**Fig. 8D**). A trend of increase in the capacity of complex IV, although it is not statistical different, was observed (**Fig. 8E**). In combination, all these findings indicated that BCO2 knockout induced alterations in mitochondrial metabolic phenotype, as shown by overactive mitochondrial respiration function in BCO2 knockout mice as compared with wild type mice.

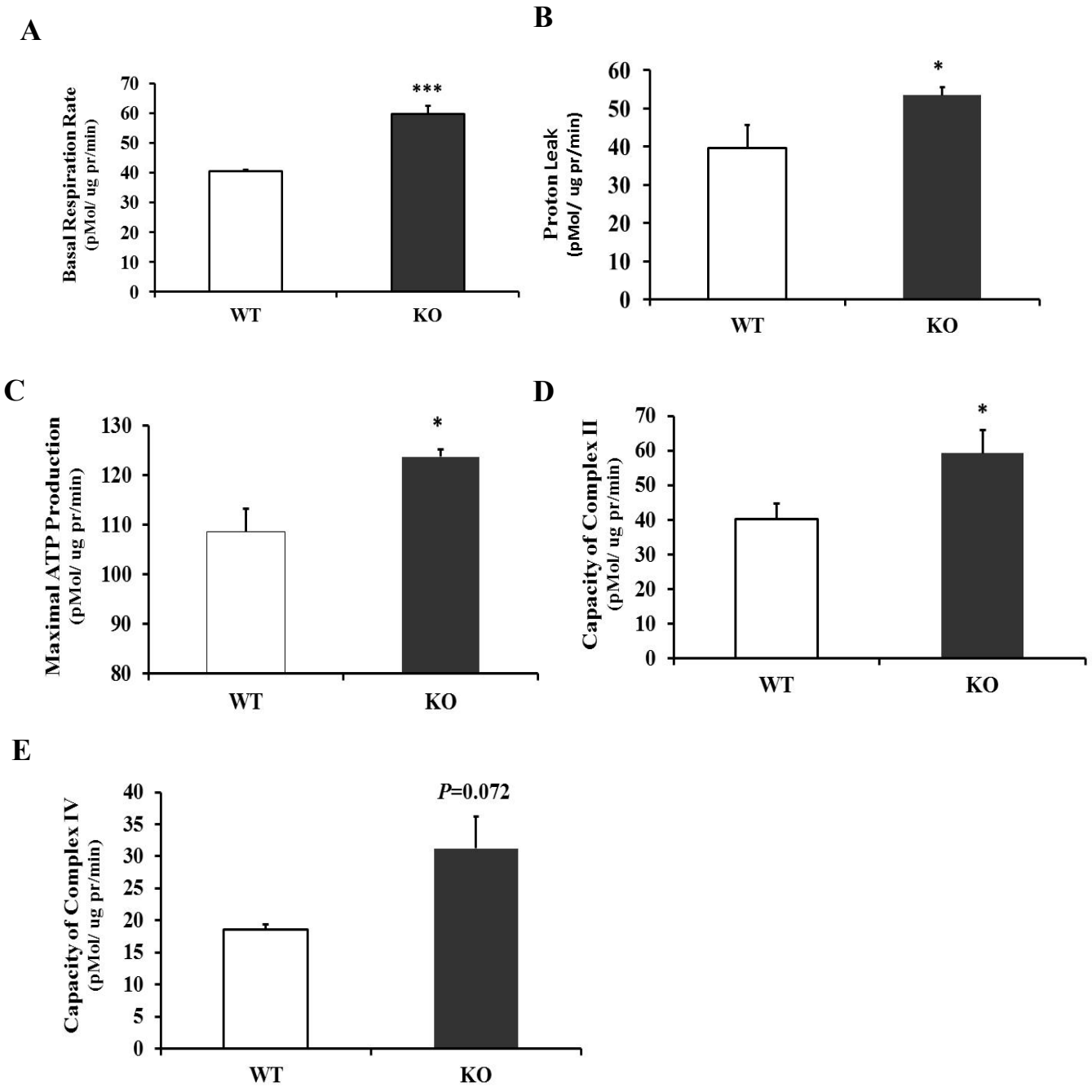
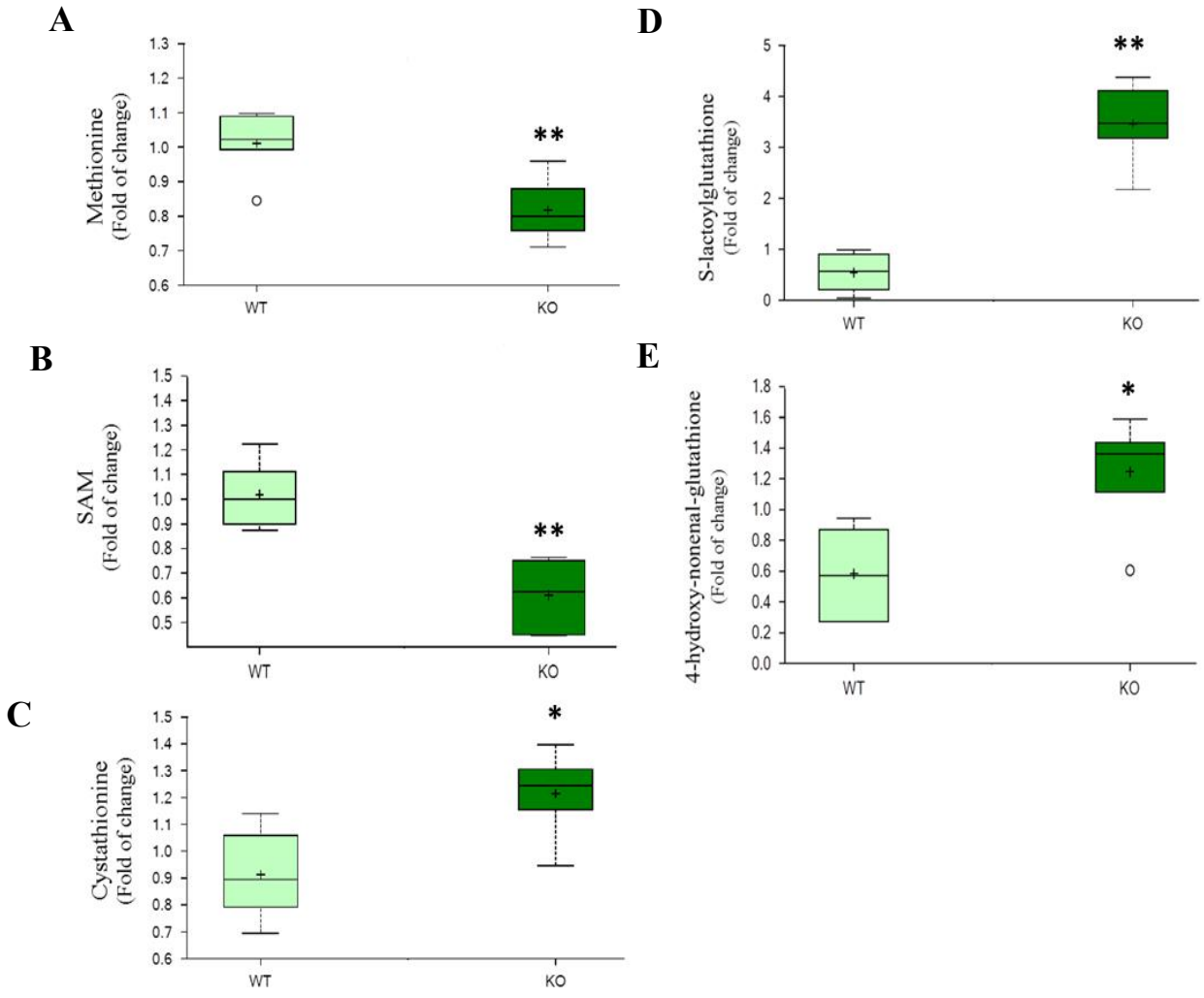


Fig. 8. Mitochondrial ATP Generation, Oxygen Consumption Rate (OCR) and Complex Activity Are Elevated with BCO2 Knockout. *A,* Basal respiration rate analysis in isolated hepatic mitochondria using Seahorse *XF*^{e} *96* analyzer. *B,* Proton leak analysis in isolated hepatic mitochondria. C, Maximal ADP production in isolated hepatic mitochondria. *D,* capacity of hepatic mitochondrial complex II was significantly increased in BCO2-knockout mice. *E,* capacity of hepatic mitochondrial complex IV was elevated but not reached significant level. n=12 technical replicates from 3 biological replicates, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Indicators of Oxidative Stress Were Elevated in BCO2 Knockout Liver

To determine whether or not BCO2 knockout induces significant metabolic alterations in the liver of mice, a global metabolomics profiling assay was performed using fresh liver tissues from both 6-week BCO2 knockout mice and age-matched wild type mice (**Fig. 9A**). A significant increase of cystathionine in knockout mice compared with wild type mice was observed. And there were also significant decreases in levels of methionine and s-adenosylmethionine (SAM) (**Fig. 9B, C**). As glutathione plays an important role in the antioxidant action, the levels of glutathione conjugates of reactive aldehydes, S-lactoylglutathione and 4-hydroxy-nonenal-glutathione (4-HNE-GS), were significantly increased (**Fig. 9D, E**). The above changes in one carbon metabolism pathway suggested that homocysteine may have been predominantly converted to cysteine rather than back to methionine. Majority of cysteine is metabolized into glutathione conjugates.

To further confirm the LC-MS/MS and global metabolomics data, immunoblotting analysis on some of the significantly changed proteins, related with oxidative stresses, were performed. NNT, which is considered as an oxidative stress marker [91], was significantly increased in BCO2 knockout mice compared to wild type mice (**Fig. 9F, G**). SOD2, an antioxidant mitochondrial enzyme involved in the defense system against reactive oxygen species (ROS) [92], was clearly up-regulated in the BCO2 knockout mice as compared with wild type mice (**Fig. 9H, I**).


A
Methionine
(Fold of change)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
**
WT
KO
D
S-lactoylglutathione
(Fold of change)
5
4
3
2
1
0
**
WT
KO
B
SAM
(Fold of change)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
**
WT
KO
E
4-hydroxy-nonenal-glutathione
(Fold of change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
KO
C
Cystathionine
(Fold of change)
1.5
1.4
1.3
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1.1
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0.9
0.8
0.7
0.6
*
WT
KO

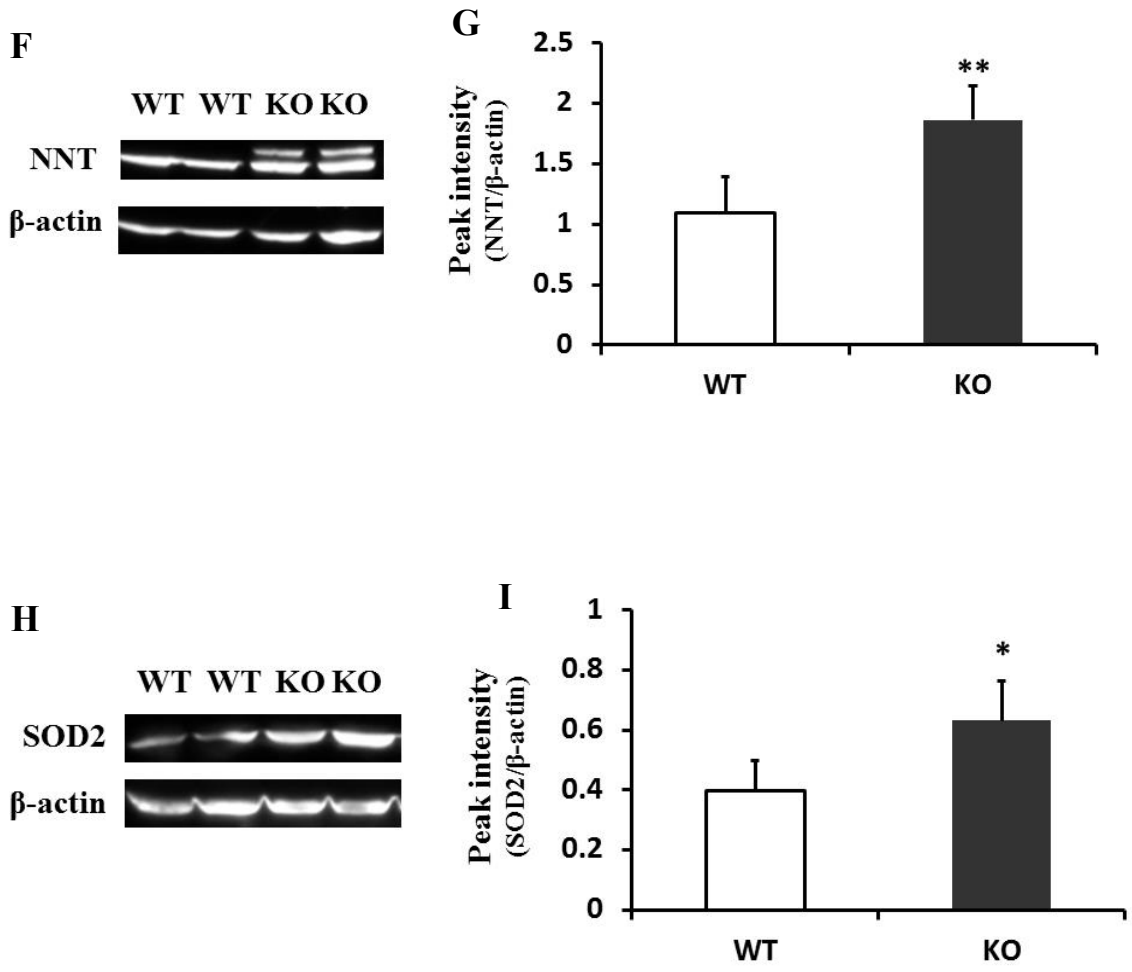

Fig. 9. Elevated Oxidative Stress Markers in BCO2 Knockout Liver. Fresh wild type and knockout liver were send to run global metabolomics analysis. *A,* Methionine level. *B,* SAM (S-adenosylmethionine) level. *C,* Cystathionine level. *D,* S-lactoylglutathione level. *E,* 4-hydroxy-nonenal-glutathione level. All these changes suggested that homocysteine may have been prominently converted into cysteine and then formed glutathione rather than betaine. Increased levels of glutathione conjugates indicated that oxidative stress was increased in KO liver. n=6 biological replicates, *p ≤ 0.05, **p ≤ 0.01. *F,* increased expression of NNT in KO mice liver (lane 3, 4) compared with WT (lane 1, 2). *G,* quantification of NNT western blot (n=6 biological replicates, **p ≤ 0.01). *H,* increased expression of SOD in KO mice liver (lane 3, 4) compared with WT (lane 1, 2). *I,* quantification of SOD western blot (n=6 biological replicates, *p ≤ 0.05).

Sterol and Lipid Metabolism Was Perturbed in BCO2 Knockout Mice

As shown in proteomic data, the metabolism of lipid was altered in BCO2 knockout mice (**Table 2.**). For instance, the decreased level of HADH indicated that the metabolism of short- or medium-chain fatty acids metabolism was altered in BCO2 knockout liver [93]. This result was also confirmed in the global metabolomics data. The level of 3-hydroxy-3-methylglutarate (HMG) was significantly increased in the BCO2 knockout mice compared with wild type mice (**Fig. 10A**). This suggested that the rate-limiting step of cholesterol synthesis, the conversion of HMG-CoA to mevalonate by HMG-CoA reductase (HMGR), was inhibited in BCO2 knockout mice leading to the alternative disposal of HMG-CoA via formation of HMG. The cholesterol precursor, squalene, was clearly decreased, supporting the conclusion that HMGR activity was reduced in BCO2 knockout mice (**Fig. 10B**). Bile acid synthesis via the 'alternative' acidic pathway involving 7-alpha-hydroxy-3-oxo-4-cholestenoate (7-HOCA) may have been more active in BCO2 knockout liver as the level of chenodeoxycholate was significantly elevated [94] (**Fig. 10C, D**). Other primary bile acids, such as the taurine-conjugate taurocholate, were reduced in BCO2-knockout liver and, secondary bile acids, such as deoxycholate, tended to be significantly decreased in livers from BCO2 knockout mice (**Fig. 10E, F**). Based on these above results, sterol and lipid metabolism was perturbed in BCO2 knockout mice.

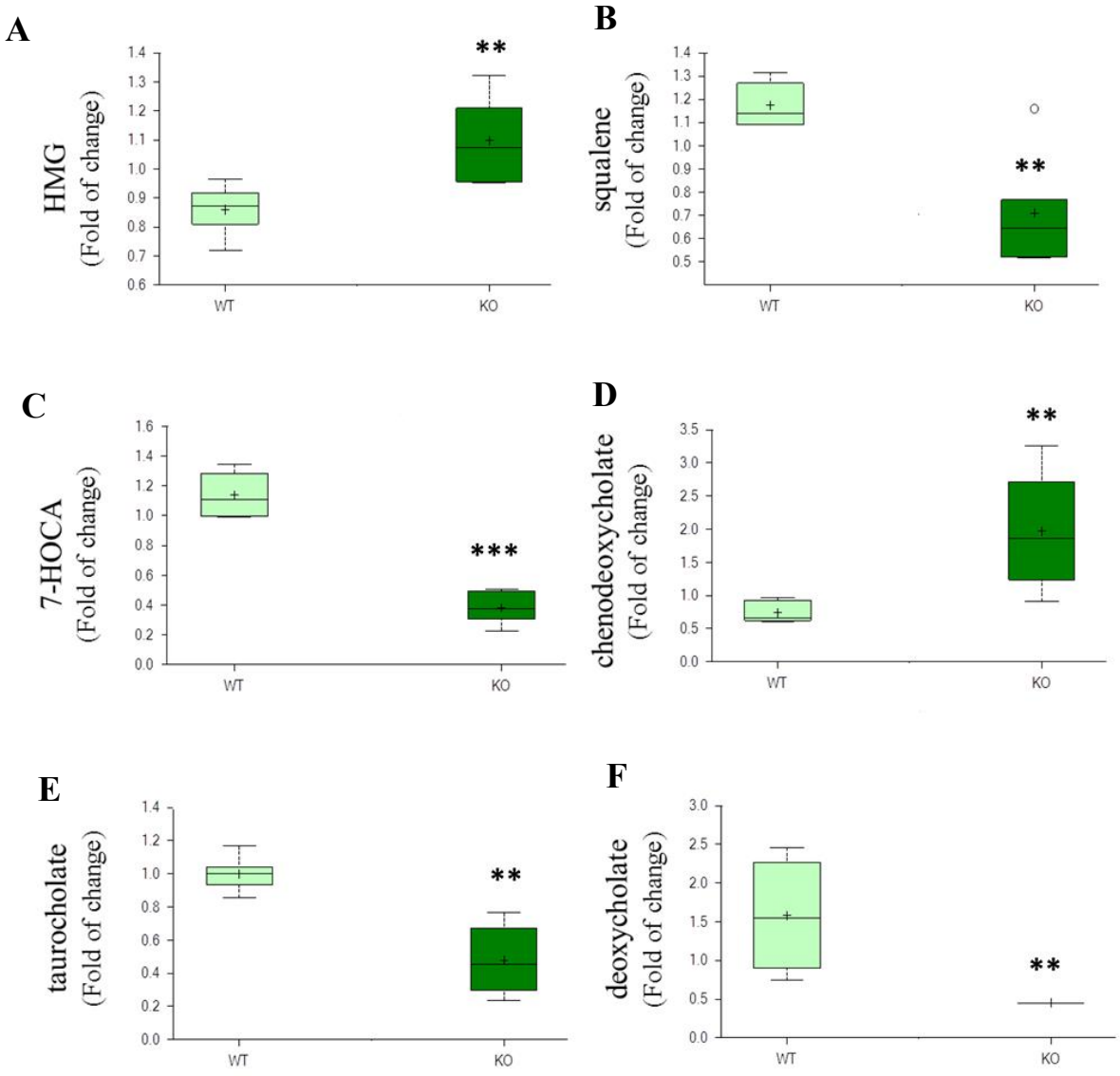

Fig. 10. Sterol and Lipid metabolism was Perturbed in BCO2 Knockout Mice. Fresh WT and KO liver were send to run global metabolomics analysis. *A,* 3-hydroxy-3-methylglutarate (HMG) level. *B,* squalene level. *C,* 7-alpha-hydroxy-3-oxo-4-cholestenonate (7-HOCA)) level. *D,* chenodeoxycholate level. *E,* taurocholate level. *F,* deoxycholate level. All these changes suggested that n=6 biological replicates, **$p \leq 0.01$, ***$p \leq 0.001$.

Energy Metabolism Was perturbed in BCO2 Knockout Mice

The unique characteristic of BCO2 knockout mice is the higher food intake compared to the wild type mice. However, there are no significant difference between the body weight in both groups.Therefore, it was hypothesized that ablation of BCO2 was associated with disturbance in energy metabolism. Therefore, pathway of energy metabolism was tested using immunoblotting analysis. The level of PPARα, which is an important transcription factor and regulator of lipid metabolism in liver [95], was significantly decreased in the BCO2 knockout mice liver compared with wild type liver (**Fig. 11A, B**). Additionally, the activation of AMPKα, which plays important roles in cellular energy metabolism [96], was significantly decreased in BCO2 knockout mice compared to wild type mice (**Fig. 11C, D**). Increased PPARα and activation of AMPKα, which are two major pathways related to energy metabolism, indicated that energy metabolism in liver was perturbed in BCO2 knockout mice.

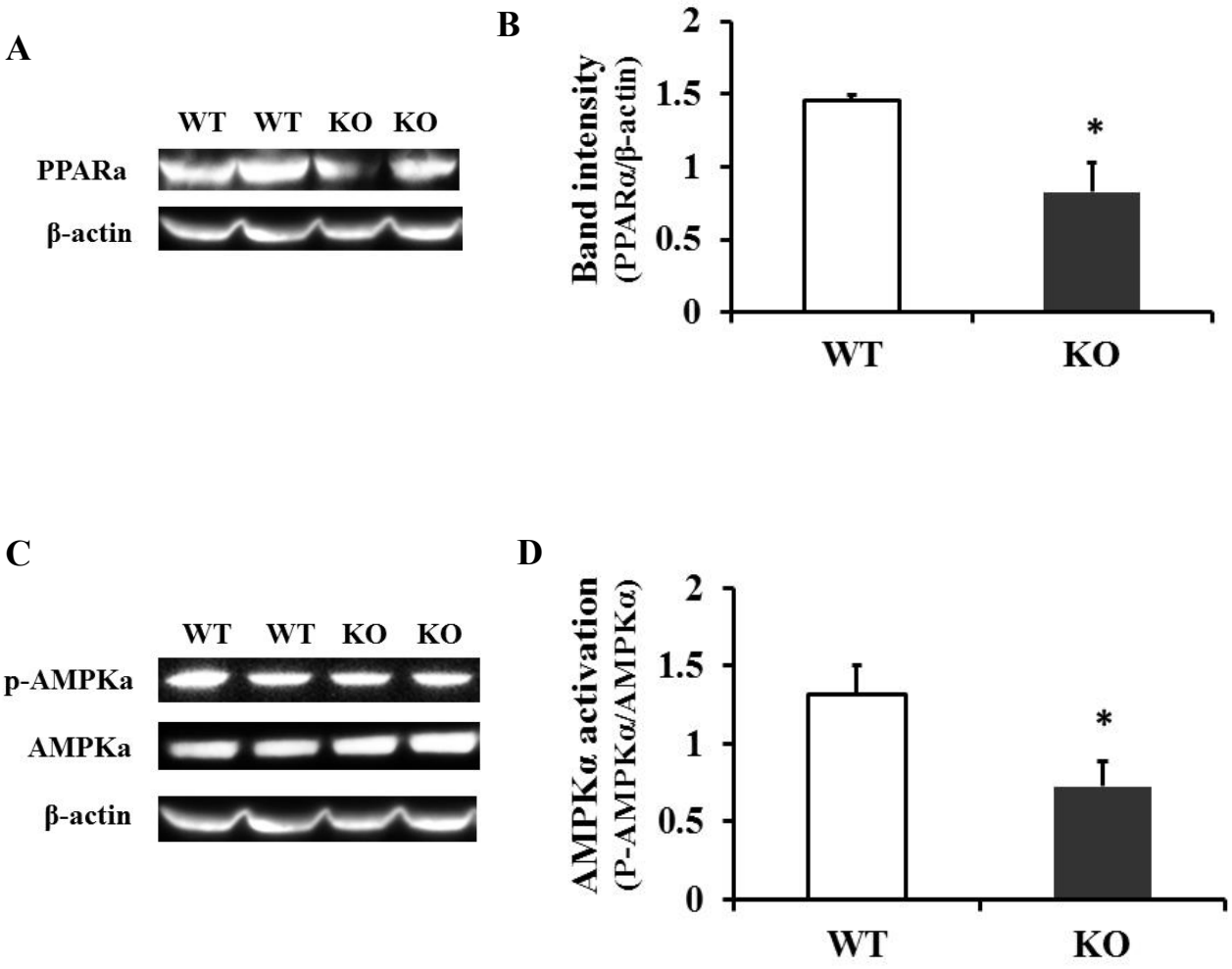

Fig. 11. BCO2 Affected Energy Metabolism in Knockout Mice. *A,* the expression of PPARα was decreased in in KO mice liver (n=6 biological replicates, lane 3,4) compared with WT (n=6 biological replicates, lane 1,2) . B, quantification of PPARα westerblot (n=6 biological replicates, *$p \leq 0.05$). *C,* the expression of phospho-AMPKα (Thr 172) was decreased in in KO mice liver (n=6 biological replicates, lane 3,4) compared with WT (n=6 biological replicates, lane 1,2). *D,* the activity of AMPKα was significantly decreased in KO mice liver compared with WT (n=6 biological replicates, *$p \leq 0.05$).

CHAPTER V

DISCUSSION

This present study demonstrated the crucial role for the inner mitochondrial membrane protein BCO2 in maintaining hepatic mitochondrial homeostasis by using BCO2 knockout mice model. We provided evidence in both mitochondrial structure and function to indicate that all these protective effects were related to BCO2. Our findings indicated that BCO2 protein expression was essential to avoid mitochondrial stress and to maintain normal mitochondrial function in liver.

BCO2 Is Not Only a Carotenoid Cleavage Enzyme

Carotenoids, divided into carotenes and xanthophyll, play important roles in human physiology, including synthesis of retinoid [97, 98], antioxidant function, and blue light filter for retina [99, 100]. Currently, some genes associated with the key factors of this metabolism pathway had been identified, including metabolizing enzymes and carotenoid transporters. BCMO1 and BCO2 are two cleavage enzymes, which are important in pro-vitamin A carotenoid metabolism. However, BCO2 also metabolize non-provitamin A carotenoids including lutein and zeaxanthin [46, 101]. A more

important role of BCO2 for carotenoid metabolism is also proved by some genetic studies. In sheep, cows and chicken, mutations or variations in the *BCO2* gene affect levels of β-carotene and xanthophyll in tissues [44, 53, 102]. Moreover, accumulation of xanthophyll such as lutein and zeaxanthin has also been found in BCO2 knockout mice [46]. Metabolism of lycopene was impaired in BCO2 knockout mice [103]. In this way, BCO2 plays more important roles in maintaining homeostasis of carotenoids in tissues compared to BCMO1.

BCO2 gene was found to correlate with altered blood concentrations of IL-18, which is a proinflammatory cytokine related with cardiovascular disease and type 2 diabetes in humans [9, 56, 104]. This association of cardiovascular disease and BCO2 was also demonstrated in mice [46]. Recent publication showed that BCO2 was enzymatically inactivated in human retina where macular pigments such as lutein and zeaxanthin are preferentially accumulated [105]. In zebrafish model, BCO2 was expressed as an oxidative stress regulator during development. Decreased expression of BCO2 resulted in anemia at the larval stage of zebrafish [60]. One recent publication showed that the expression of BCO2 was inhibited in the liver of obese mice and in the diabetic retina [11]. The expression of BCO2 was also found to inversely relate to AMPKα-mediated mitochondrial stress and autophagy in obese mice [33]. Therefore, these findings suggest that BCO2 may have other novel roles rather than only as a carotenoid cleavage enzyme.

BCO2 Knockout Causes Hepatic Mitochondrial Dysfunction

Mitochondrial dysfunction is considered as one major underlying symptom linking obesity with diabetes and metabolic syndromes [106, 107]. Focusing on the changes in mitochondrial proteins, pathways, and activities can serve as a reference to better understand and finally provide some therapeutic targets for treating and preventing obesity, diabetes and associated metabolic syndromes. With these purposes in mind, we used the proteomic approach to define the changes of protein expression in mouse hepatic mitochondria from the BCO2 knockout mice.

Our results illustrated that BCO2 knockout triggered alterations in various metabolic pathways including fatty acid oxidation, amino acid metabolism, TCA cycle and oxidative phosphorylation. The top two pathways that were significantly affected by BCO2 deficiency were mitochondrial dysfunction and mitochondrial oxidative phosphorylation. When the selected proteins were analyzed by IPA, we found that the protein level of mitochondrial complex I, III and IV were predicted to decrease in the BCO2 knockout mice compared with wild type mice. Interestingly, we found the expression of cytochrome C was increased in the BCO2 knockout mice. It is possible that mitochondria try to compensate for the decreased expression levels of mitochondrial complexes by increasing the level of cytochrome C, which functions as the electron transporter between mitochondrial complex III and IV [108]. The increased level of cytochrome C will elevate the amount of electrons, which can be transported between the complexes. This kind of compensatory mechanism will help to maintain mitochondrial homeostasis. In addition to these, the increased levels of carotenoids metabolism protein, such as CYP1A2, CYP2D9, CYP7B1 and CYP2C40, indicate that the vitamin A

metabolism is distinctly up-regulated. The expression of mitochondrial transporter, such as TIMM50, VDAC1 and VDAC3, are down-regulated under BCO2 knockout condition.

The proteomic data showed us the overall pathways that were altered by deletion of BCO2. In order to identify the detailed mechanisms contributing to these altered pathways, we also used electron microscopy to observe the changes in the mitochondrial structure. As showed in Fig.6, we found that mitochondrial number and shape was changed in the BCO2 knockout mice compared with the wild type mice. The most obvious difference between the mitochondria from BCO2 knockout liver and wild type liver is that mitochondrial number was increased and the mitochondrial size was decreased in the BCO2 knockout mice. More glycogen granules and lipid droplets were also observed in the BCO2 knockout mice. Glycogen, the storage of glucose, is found in most tissues in the body, including liver and skeletal muscle. The major function of glycogen is to store excess glucose, either for cells own survival or for other cells [109]. In BCO2 knockout liver, the number of glycogen granules was greater than the wild type indicated that the glycogen synthesis was elevated or the usage of glycogen was inhibited [110]. Based on our lab's high fat (45% energy from fat) dietary intervention study, in BCO2 knockout mice, the rate of gluconeogenesis was increased compared to wild type mice. This is one possibility to explain the increased numbers of glycogen granules in BCO2 knockout liver. What's more, the respiratory quotient (RQ) was increased in BCO2 knockout mice. The RQ for carbohydrate is higher than fat. All these data indicate that the BCO2 knockout mice prefer to use carbohydrate instead of lipid for energy [111, 112]. This could also be confirmed by the evidence that the accumulation of lipid in the liver, indicating the knockout mice could not metabolize the lipid properly. Increased

mitochondrial copies may be a possible structural remodeling method to accommodate the lower protein levels of oxidative phosphorylation pathway [113].

Electron microscopic analysis of the hepatic mitochondria revealed that BCO2 knockout promoted mitochondrial change to a different status with decreased size and increased number. Furthermore, hepatic mitochondrial functional analysis was also conducted. The results demonstrated that the basal respiration rate and proton leak were increased in BCO2 knockout mice *versus* wild type mice. The ETC creates a proton gradient that can be used to generate the production of ATP. However, in knockout mice, the proton leak was increased, which indicated that more protons were leaked back to mitochondrial matrix and fewer protons could be used to generate ATP production. As the proton gradient was perturbed, the efficiency of ETC was decreased [114, 115]. In this way, we propose that in BCO2 knockout mice the hepatic mitochondria was trying to maintain the ETC homeostasis via up-regulating the basal respiration rate to compensate for the decreased ATP production caused by proton leak. The condensed hepatic mitochondria together with increased basal respiration rate in BCO2 knockout liver strongly indicated that mitochondrial ETC are energetically active. It has been showed that the structural transition between “orthodox” mitochondria and “condensed” mitochondria is a reflection of energy utilization in mitochondria [113]. As we observed the decreased protein expression involved in fatty acid metabolism concomitant with the condensed mitochondria, it can be hypothesized that the BCO2 knockout hepatic mitochondria do not preferentially utilize fatty acids for ATP production.

As shown in proteomic data, the proteins in oxidative phosphorylation pathway, such as complex I, III and IV, were decreased in BCO2 knockout liver. However, the

basal respiration rate was increased in BCO2 knockout liver. Therefore, we carried out "electron flow" experiment using freshly isolated hepatic mitochondria from BCO2 knockout mice. Increased activities of complex II and IV in BCO2 hepatic mitochondria were found and these results were consistent with the increased basal respiration rate and the increased level of cytochrome C. Furthermore, "coupling assay" also showed that the ATP production was increased in the BCO2 knockout liver *versus* wild type.

The other novel observations in this study are the changed markers of mitochondrial metabolism in BCO2 knockout liver. For instance, some amino acids, such as leucine, and their intermediates, such as 2-aminoadipate derived from lysine or tryptophan degradation, whose disposal are dependent on the mitochondria were significantly decreased in BCO2 knockout compared to wild type liver. However, 4-methyl-2-oxopentanoate, which is the deamination product of leucine, was elevated. Homocitrulline, which is formed when lysine is substituted for ornithine in the mitochondrial reaction catalyzed by ornithine transcarbamylase (OTC), was increased in BCO2 knockout liver. All these changes suggest that the oxidation of amino acids in mitochondria, such as leucine, tryptophan, and lysine, was inhibited in BCO2 knockout livers. Mammalian/mechanistic target of rapamycin (mTOR), an important indicator of mitochondrial autophagy [116, 117], was significantly increased in BCO2 knockout mice compared to wild type mice. This indicates that more abnormal mitochondria produced and needed to clear in the BCO2 knockout mice. Mitochondrial dysfunction is one source to produce abnormal mitochondria. Therefore, the increase level of mTOR showed that mitochondrial dysfunction happened in BCO2 knockout mice.

BCO2 Knockout Leads to Increased Oxidative Stress

Oxidative stress, caused by an imbalance between the systemic production of reactive oxygen species (ROS) and systemic cellular redox ability to detoxify these reactive intermediates, is a key factor in several diseases including obesity, aging and diabetes [16, 118, 119]. One major source of ROS production is from mitochondrial oxidative phosphorylation pathway. The leakage of electrons back into matrix instead of transferring among mitochondrial complexes contributes to the production of ROS, which can be quenched by mitochondrial SOD or the protons leaked into mitochondrial matrix [120]. The proton leak was increased in the BCO2 knockout mice, indicating that more ROS was produced in the mitochondrial matrix. The increased level of mitochondrial SOD also indicated that ROS production was elevated in the mitochondrial matrix.

Aside from the increased production of ROS, the global metabolon data also showed that oxidative stress was increased in the liver. Levels of sulfur-containing amino acids such as methionine, SAM and SAH, which are important for one carbon transfer reactions, were significantly decreased in BCO2 knockout mice [121, 122]. This finding indicated that the remethylation pathway, which is catalyzed by methyltransferases, was inhibited in BCO2 knockout mice. However, increased level of cystathionine showed that homocysteine may be predominantly converted to cystathionine rather than back to methionine. The increased cystathionine was preferentially metabolized into glutathione or glutathione conjugates. In this way, an elevated level of glutathione conjugates of reactive aldehydes, such as S-lactoylglutathione and 4-HNE-GS, indicated that oxidative stress was greater when BCO2 expression was suppressed. Therefore, more glutathione

was needed to neutralize these reactive compounds, including ROS [123, 124]. Thus, the findings from mitochondrial functional analysis, immunoblot analysis and global metabolomics analysis appeared to confirm that increased level of oxidative stress was presented in BCO2 knockout mice liver.

BCO2 Knockout Disturbs Sterol and Lipid Metabolism

According to proteomic data, lipid metabolism was altered in the liver of BCO2 knockout mice. In BCO2 knockout mice, the HADH level was significantly decreased. HADH is an important lipid metabolism enzyme, which catalyzes the metabolism of short-and medium-chain fatty acids [93]. Decreased expression of HADH may indicate that short-and median-chain fatty acids metabolism is inhibited in BCO2 knockout liver. However, the level of ECT1, which catalyzes the conversion of trans- or cis-double bonds of fatty acids at γ-carbon to trans-double bone at β-carbon and plays important roles in the unsaturated fatty acids metabolism, was elevated. Combined with the elevated HADH level, BCO2 knockout mice shifted preference to unsaturated fatty acids from short- and/or medium-chain fatty acids [125].

The global metabolomics data showed similar results, pointing out that the markers associated with sterol metabolism and the conversion of cholesterol to primary and secondary bile acids were perturbed in liver of BCO2 knockout mice. HMG was found to increase significantly in BCO2 knockout mice, indicating that the limiting step of cholesterol synthesis, which is the conversion of HMG-CoA to mevalonate by HMGR, was inhibited. In this way, ablation of BCO2 will disrupt the cholesterol synthesis. The cholesterol precursor, squalene, was clearly decreased, supporting the conclusion that

HMGR activity was reduced in BCO2 knockout mice. In our lab's high fat dietary intervention study, the serum cholesterol levels was decreased after 8-week high fat diet treatment (45% energy from fat), however, the food intake was much higher in BCO2 knockout mice compared wild type mice. The animal study just confirmed the result from chow diet intervention that lipid metabolism was disrupted in BCO2 knockout mice.

There are two major pathways for bile acid synthesis, including both classical and alternative pathway. The expression of 7-HOCA, which mediates the "classical" bile acid synthesis pathway, was significantly decreased in BCO2 knockout liver. What's more, the primary bile acids synthesized in "classical" bile acid synthesis pathway, such as the taurine-conjugate taurocholate, were reduced in BCO2 knockout liver. All of these data show that the "classical" pathway is inhibited in BCO2 knockout mice. However, the levels of chenodeoxycholate and α- muricholate and β-muricholate, which synthesized from "alternative" bile acid synthesis pathway, were elevated. Therefore, all the findings indicate that the BCO2 knockout mice prefer to use "alternative" bile acid synthesis pathway instead of the "classical" bile acid synthesis pathway. Additionally, the secondary bile acids, such as deoxycholate, tended to be significantly decreased in the liver of BCO2 knockout mice. All of these changes indicated that sterol and lipid metabolism were dysregulated in BCO2 knockout liver.

BCO2 Is Essential for Maintaining Energy Metabolism

When BCO2 knockout mice fed with high fat diet (45% kcal from fat), the food intake was much higher in BCO2 knockout mice compared to wild type mice. However, the body weight gain was not significantly different between the two groups. In this way,

we hypothesized that the energy metabolism was perturbed in BCO2 knockout mice. In this present study, we found that the level of PPARα, which is an important transcription factor and regulator of lipid metabolism in liver, was significantly decreased in the BCO2 knockout mice liver compared with wild type liver [126]. This indicated that the BCO2 knockout mice could not utilize lipid properly. Additionally, the activation of AMPKα was also decreased in BCO2 knockout mice compared to wild type mice. Phospho-AMPKα, which is the active form of AMPKα, plays important roles in the cellular energy metabolism [127]. Since the energy metabolism was dysregulated, BCO2 knockout mice could not produce enough energy to maintain whole-body homeostasis. In this way, the food intake of BCO2 knockout mice was significantly higher than wild type mice as the efficiency of energy production was lower. There still exists other possibility that BCO2 might directly impact the function of hypothalamus, which is the regulation center of eating behaviors [128]. Future studies should be conducted to figure out the underlying mechanism of the association between BCO2 and food intake behavior.

In conclusion, the results presented here indicate that decreased expression of BCO2 will decrease the ETC efficiency via increasing the proton leak and basal respiration rate. Therefore, knockout of BCO2 will lead to mitochondrial dysfunction, which contributes to the increased cellular levels of oxidative stress. Numerous publications have demonstrated that mitochondrial dysfunction and cellular oxidative stresses are important factors in the pathogenesis of obesity. In this way, BCO2 might play roles in the development of obesity [16, 17, 26, 129]. However, we are still at the early stage of understanding importance of BCO2 in obesity development. Future studies

should focus on animal studies to figure out the detail mechanism of BCO2 on obesity development.

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